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Characterization of *Botrytis cinerea* causing grape bunch rot in Chinese vineyards

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Abstract

Botrytis is an important plant pathogenic genus causing bunch rot of grape berries and other hosts worldwide. A survey of vineyards in 14 provinces of China was carried out to identify the causal agent of Botrytis bunch rot. A phylogenetic analysis based on a combination of RPB2, HSP60, G3PDH and Nep1, Nep2 loci, coupled with morphology, showed that *Botrytis cinerea* is the main causal agent of grape bunch rot disease in China. Pathogenicity tests showed that wounded grape berries are more susceptible to *Botrytis cinerea* infection than non-wounded berries. This paper contributes to the fact that Nep1 and Nep2 gene regions provide a higher resolution in distinguishing species of *Botrytis*.

Key words – Gray mold – Grapevine – Nep1 – Nep2 – Pathogen

Introduction

Grapevine (*Vitis vinifera* L., family *Vitaceae*) is one of the most widely cultivated crops worldwide. China holds the first position among Asian countries in cultivating wine grapes with 580,000 hectares (IOV 2013). As this crop has become more widely grown in China, many fungal, bacterial, and viral diseases have become major problems for grapevine cultivation, especially fungal diseases (Jayawardena et al. 2014).

Botrytis Pers. (*Sclerotiniaceae*, Ascomycota) was introduced by Micheli (1729) and later validated by Persoon (Jarvis 1980). It was one of the first described genera of fungi (Hyde et al. 2014). It is recommended to use the name *Botrytis* over its sexual morph *Botryotinia*, since *Botrytis* is the oldest and the most commonly used name (Kirk et al. 2013, Hyde et al. 2014). *Botrytis* species are important pathogens of many agro-economic crops such as grapes, kiwifruit, lettuces, tomato, strawberry and ornamental crops (Giraud et al. 1999, Fournier et al. 2005, Staat et al. 2005). Species of *Botrytis* cause gray mold disease on more than 200 mostly dicotyledonous plant species (Williamson et al. 2007). Gray mold infection leads to serious losses in yield and quality under humid conditions on numerous crops, particularly fruits, vegetables and ornamental flowers (Leroch et al. 2012). Gray mold caused by *Botrytis* species is probably the most important pre- and

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postharvest disease of grapevine. Northern Chinese vineyards are frequently affected by gray mold (Chen et al. 2006) (Fig 1).

In China, the causal agent of gray mold has long been identified as *B. cinerea* (Du et al. 1992, Lei et al. 2005, Chen et al. 2006, Zhou et al. 2014), which is considered to be a typical heterothallic fungus. *Botrytis pseudocinerea* A.S. Walker et al. and *B. sinoviticola* J. Zhang et al. are also recognized to cause bunch rot of grapevine in China (Zhou et al. 2014). The aim of the current paper is to identify and characterize the *Botrytis* species occurring on grapevine in China using both morphological and molecular data. This is the eleventh contribution in a series of papers re-assessing the genera and species of fungi causing grapevine diseases in China (Yan et al. 2013, 2014, Dissanayake et al. 2014, Jayawardena et al. 2014, Liang et al. 2015).

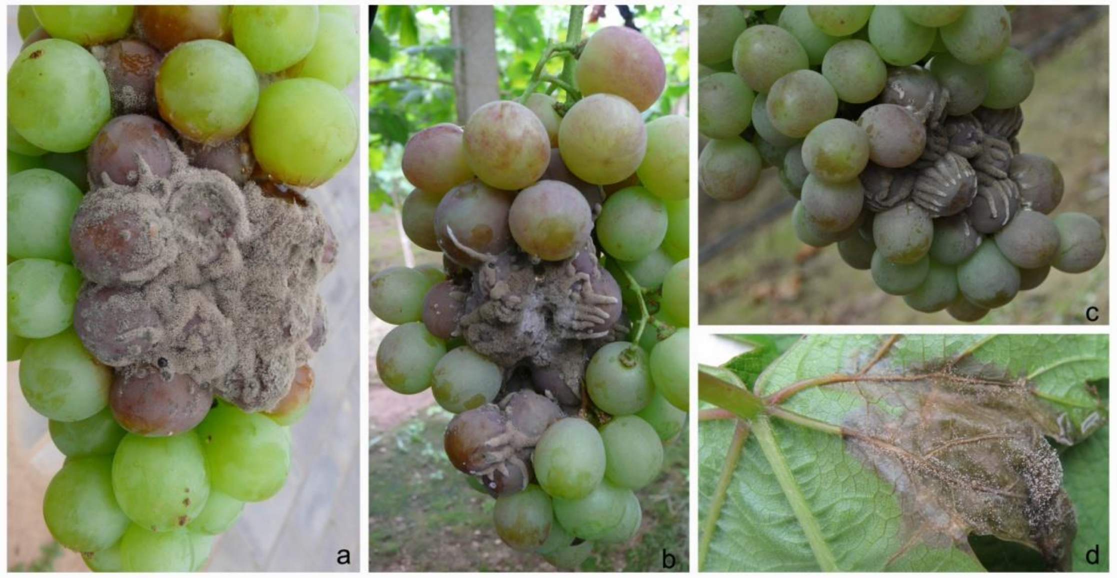

Fig. 1 – Disease symptoms of *Botrytis* on grapes in a vineyard. a–c *Botrytis* on grape bunch. d *Botrytis* on a leaf

Materials & Methods

Isolation and Identification

Isolates were collected from Beijing, Gansu, Guangxi, Hebei, Henan, Hubei, Hunan, Jiangsu, Liaoning, Ninxia, Shandong, Shanghai, Shanxi, Tianjin and Zhejiang provinces of China. Diseased grapevine samples were collected and placed in separate plastic bags with sterilized tissues dipped in distilled water to maintain humidity. Samples were surface sterilized with 75% ethanol for 1 minute and then rinsed three times with water. The fungi, present on samples were isolated by single spore culture technique (Chomnunti et al. 2014). The pure isolates obtained were cultured on potato dextrose agar (PDA) plates with sterilized filter paper pieces and incubated for 7–10 days at 25 °C. Cultures on the filter paper pieces were dried on sterilized filter paper and stored at –20 °C. The morphology of fungal colonies was recorded following the method of Hennebert (1963), Zhang et al. (2010 a, b) and Zhou et al. (2014). Fungal mycelia and spores were observed and photographed using a Leica DM5500B microscope.

Molecular Phylogeny

DNA extraction, PCR amplification, and DNA sequencing

Isolates were transferred to 90 mm diameter Petri dishes containing PDA. Total genomic DNA was extracted from fresh cultures (500 mg), scraped from the margin of a colony on a PDA plate incubated at 25 °C for 7–10 days. The method of Zhang et al. (2010 a, b) was used for DNA extraction with minor modifications. The RPB2, HSP60, GPDH, Nep1, Nep2 genes were amplified using primer pairs RPB2for+/RPB2rev+ (Staats et al. 2005), HSP60for+/HSP60rev+ (Staats et al. 2005), G3PDHfor+/ G3PDHrev+ (Staats et al. 2005), Nep1(-207)for/Nep1(+1124)rev (Staats et al. 2007 a, b), Nep2(-200)for/Nep2(+1147) rev (Staats et al. 2007 a, b), respectively. The PCR was performed in a BIORAD 1000^{TM} Thermal Cycler in a total volume of 25 µl. The PCR mixtures contained TaKaRa Ex-Taq DNA polymerase 0.3 µl, 12.5 µl of 2 × PCR buffer with 2.5 µl of dNTPs, 1 µl of each primer, 9.2 µl of double-distilled water and 100–500 ng of DNA template. The thermal cycling program followed Li et al. (2012). The PCR products were verified by staining with ethidium bromide on 1.2% agarose electrophoresis gels and purified according to the manufacturer's instructions of a Qiangen purification kit (Qiagen, USA) and for ligation PMD – 18T vector (Takara Company, Dalian, China) was used. The DNA sequences of RPB2, HSP60, G3PDH, Nep1 and Nep2 regions generated in this study were submitted to GenBank.

Phylogenetics analysis

DNAStar and SeqMan were used to obtain consensus sequences from data generated from forward and reverse primers. Single and combination of multi-locus datasets of the gene regions were aligned using Clustal X1.81 (Thompson et al. 1997). The sequences were further aligned using default settings of MAFFT v.7 (Katoh & Toh 2008; http://mafft.cbrc.jp/alignment/server/) and manually adjusted using BioEdit (Hall 1999) where necessary. A maximum parsimony analysis (MP) was performed using PAUP (phylogenetic analysis using parsimony) v. 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded and gaps were treated as missing data. Trees were inferred using the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1,000 random sequence additions. Maxtrees were set to 5,000 branches of zero length were collapsed, and all multiple parsimonious trees were saved. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for trees generated under different optimality criteria. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications resulting from maximum parsimony analysis (Hillis & Bull 1993). The Kishino-Hasegawa tests (Kishino & Hasegawa 1989) were performed in order to determine whether the trees inferred under different optimality criteria were significantly different.

In addition, Bayesian inference (BI) was used to construct the phylogenies using Mr. Bayers v. 3.1.2 (Ronquist et al. 2012). MrModeltest v. 2.3 (Nylander 2004) was used to carry out statistical selection of best-fit models of nucleotide substitution. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation. The first 2,000 trees, representing the burn-in phase of the analyses, were discarded and the remaining 8,000 trees used for calculating posterior probabilities (PP) in the majority rule consensus tree. Phylogenetic trees were drawn using Treeview (Page 1996). The alignments and trees are deposited in TreeBASE (www.treebase.org/treebase/index.html). The fungal strains that were used for this study are listed in Table 1.

Pathogenicity test

Pathogenicity tests were conducted on healthy fruit of *Vitis vinifera* cv. Red Globe that were uniform in size and lacking visible disease symptoms. The experiment comprised 40 fruits (including 10 controls). The fruits were washed with tap water and then disinfected in 1% sodium hypochlorite for 1–3 minutes. Disinfected fruits were washed three times with distilled, sterilized water and then dried with sterilized filter paper, before inoculation. Inoculations used wound and non-wound inoculation methods. A sterile needle was used to prick the fruit wall, and a 5 mm diam. PDA agar disk was placed on the wound. The non-wound inoculation method involved placing PDA agar disks (5 mm diam.) on the fruit without pricking the fruit wall. Controls were inoculated with a sterile agar plug. All the fruit were incubated in a moist chamber at ± 95% relative humidity and at 28 °C until symptoms appeared. Re-isolations were made to confirm Koch's postulates.

Data analysis

Data from the pathogenicity tests were analyzed using Minitab, V.15.1.1.0 (Minitab release 15.1.1.0, Minitab, Boston, MA, USA). Treatment means were compared using Turkeys' test at the 5 % significance level.

Results

Isolation of fungi

Botrytis species were isolated from berries with grey-brown mycelium and from reddish brown necrotic lesions (Fig. 1). Twenty-seven isolates were obtained from disease samples and deposited in Beijing Academy of Agriculture and Forestry Sciences culture collection. Isolates were obtained from 16 grape varieties including three traditional varieties (cv. brier grape, cv. JinXiangyu, cv. Shanghai pei one).

Phylogenetic analysis

Phylogenetic trees were constructed using combined sequence data from RPB2, HSP60 and GPDH for our isolates of *Botrytis* and with those that originated from Hyde et al. (2014), Zhou et al. (2014) (Table 1). A separate phylogenetic tree was constructed using combined sequences of Nep1 and Nep2 for our isolates and with those that originated from Hyde et al. (2014). Maximum-parsimony and Bayesian inference analyses produced nearly identical topologies (Bayesian trees are not shown).

The combined gene alignment comprised 63 taxa and 2939 characters including gaps (GADPH: 1–893, HSP60: 894–1854, RPB2: 1855–2939), with *Sclerotinia sclerotiorum* (strain 484) as the out group taxon. Parsimony analysis indicated that 2261 characters were constant, 321 variable characters uninformative and 357 characters parsimony-informative. Parsimony analysis resulted in ten most parsimonious trees, one of them is shown (Fig. 2) (TL = 1109, CI = 0.710, RI = 0.908, RC = 0.644, HI = 0.290).

Combined gene analysis of Nep1 and Nep2 comprised 57 taxa and 1581 characters including gaps. Parsimony analysis indicated that 999 characters were constant, 190 variable characters uninformative and 392 characters parsimony-informative. Parsimony analysis resulted in ten most parsimonious trees, one of them is shown (Fig. 3) (TL = 1071, CI = 0.657, RI = 0.882, RC = 0.580, HI = 0.343).

Bootstrap support (BS) values and Bayesian posterior probabilities (PP) (equal to or above 50% based on 1,000 replicates) are shown near the nodes. Combined gene analysis resulted in clustering of our isolates with *B. cinerea* with strong support.

Botrytis cinerea Pers., Ann. Bot. (Usteri) 1: 32 (1794)

Index Fungorum number: IF217312; Faces of fungi number: FoF03822

Synonyms in Index Fungorum (2018)

At the onset of ripening (verasion), grapes are infected either directly through the epidermis or through wounds. The fungus then progressively invades the entire cluster. Infected berries first appear soft around the lesion, with ruptured skin that is covered with grey mycelium. Later, the skin appears to be sunken and watery around the lesion, and covered with grey mycelium and conidial mass (Fig. 1). Twenty-seven isolates obtained from the diseased samples in this study were identified as *B. cinerea*. For morphology (Fig. 4) refer to Aktaruzzaman et al. (2017).

Pathogenicity test

Lesions resembling initial symptoms were observed after 6 days on leaves. The lesion obtained by wound inoculation method were longer ($\overline{x}$ = 2.07 cm) than the lesions obtained by non-wound inoculation method ($\overline{x}$ = 0.52 cm). *Botrytis cinerea* was successfully re-isolated from the wounded and unwounded grapevine fruit. Control fruit remained healthy (Fig. 5).

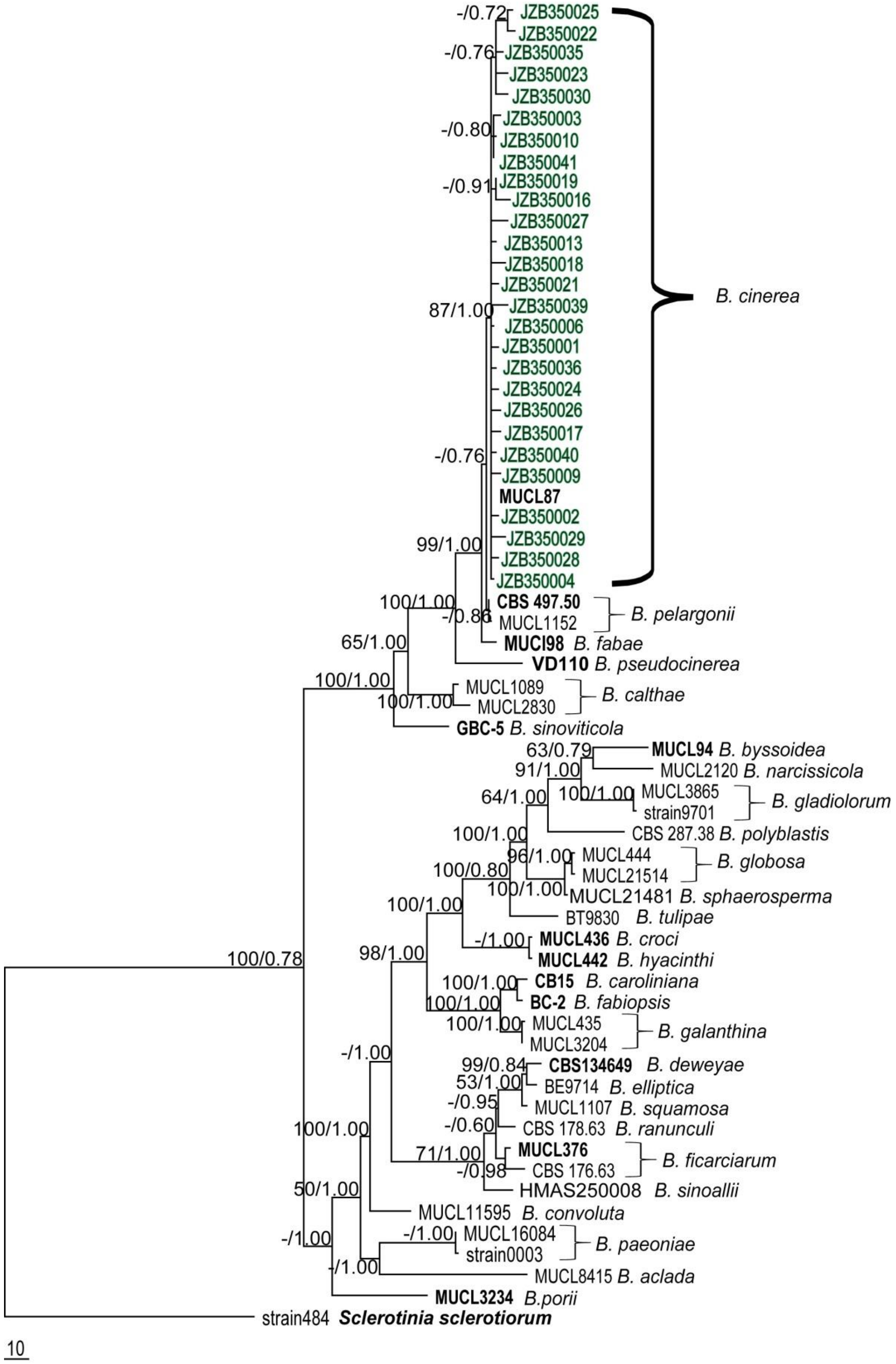

Fig. 2 – Maximum parsimonious tree obtained from a heuristic search of the combined RBP2, GAPH and HSP60 sequence alignment. Bootstrap support values above 50% and Bayesian posterior probability values above 0.5 are shown near the nodes. *Sclerotinia sclerotiorum* (strain 484) is used as outgroup. Isolates obtained in this study are shown in green. Ex-type and ex-epitype strains are in bold.

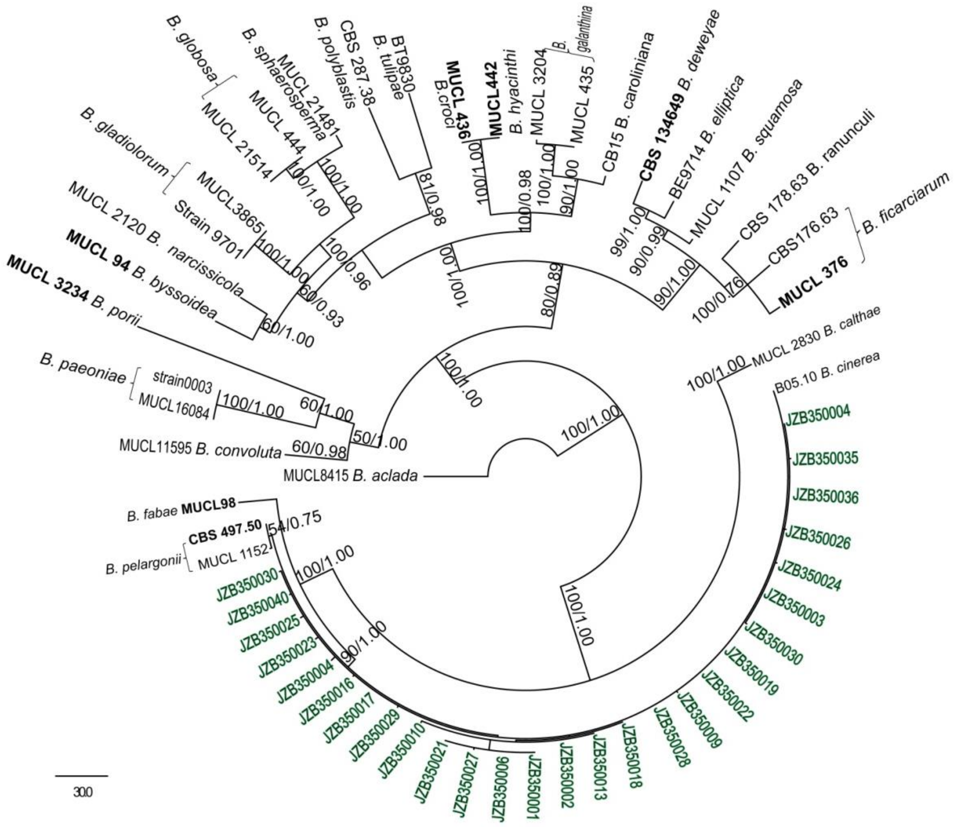

Fig. 3 – Maximum parsimonious tree obtained from a heuristic search of the combined Nep1 and Nep2 sequence alignment. Bootstrap support values above 50% and Bayesian posterior probability values above 0.5 are shown near the nodes. Isolates obtained in this study are shown in green. Ex-type and ex-epitype strains are in bold.

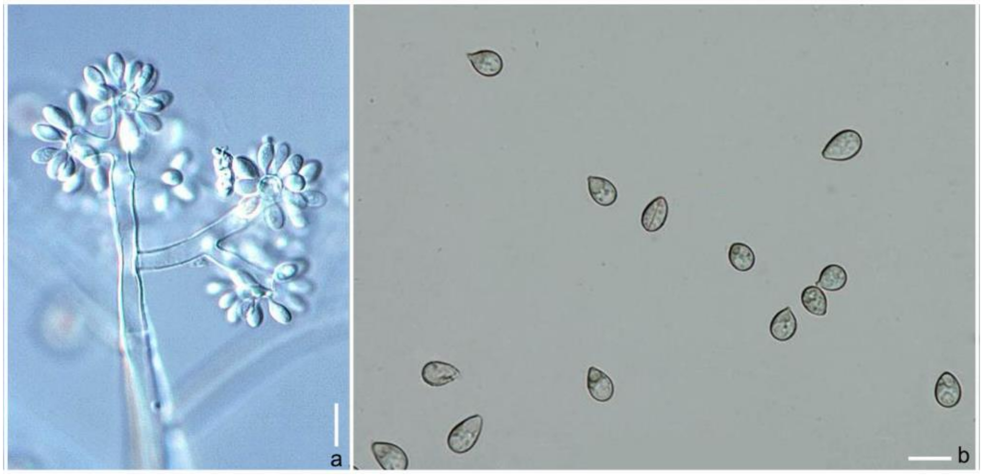

Fig. 4 – *Botrytis cinerea*. a Many rounded conidia are budded off at the branched ends of the long upright conidiophores. b Multinucleate, ellipsoid to obovoid conidia. Scale bars: = 20µm.

Discussion

Botrytis cinerea causes leaf blight, shoot blight, inflorescence blight and bunch rot of grapevines and is also the agent of noble rot on grapevine, which is used for the production of sweet wine (Fournier et al. 2005, Wang et al. 2010, Zhou et al. 2014). Among these diseases, Botrytis bunch rot is one of the major fruit rot diseases of grapevines (Cappellini et al. 1986, Chen et al. 2006, Zhou et al. 2014). *Botrytis* species are responsible for reducing the quality and quantity of grapes by premature cluster drop and postharvest bunch rot (Morris 1985). The fungus infects the grapes directly through the epidermis or through old wounds and progressively invades the clusters (Zhou et al. 2014). Compact clusters are the most susceptible for Botrytis bunch rot. During dry weather, infected berries tend to dry out, but in wet weather, brownish or grey mold forms on the surface of the infected berries (Nelson 1951a,b, Vail & Marios 1991, Vail et al. 1998). In wine production, Botrytis bunch rot will result in off-flavored wines due to the modified chemical composition of diseased berries (Gadoury et al. 2007).

Botrytis cinerea is the best-studied species in the genus (Williamson et al. 2007, Staats et al. 2007a, b) and was recently selected as the second most important plant pathogenic fungal species in the world (Dean et al. 2012). It is the most common pathogen causing Botrytis bunch rot, although population genetic studies have demonstrated that it is a species complex (Giraud et al. 1997, 1999, Albertini et al. 2002, Fournier et al. 2003, 2005, Walker et al. 2011, Hyde et al. 2014). Giraud et al. (1997) proposed two varieties in the *B. cinerea* complex based on the presence or absence of the transposable elements Boty and Flipper; *B. cinerea* var. *transposa* and *B. cinerea* var. *vaccuma*. In vineyards, particularly at harvest, *transposa* strains are predominant (Esterio et al. 2011, Giraud et al. 1997, Muñoz et al. 2002, Samuel et al. 2012), while *vacuma* strains are detected mainly on floral residues and strongly decrease during summer (Vercesi et al. 2014). The use of a Bc-*hch* population marker also suggested the existence of two groups (I and II) in *B. cinerea* populations (Fournier et al. 2003). Multi-gene phylogenetic analysis (Staats et al. 2005, Walker et al. 2011, Hyde et al. 2014) also support the two-group classification proposed by Fournier et al. (2003). Walker et al. (2011) defined the Group I isolates of *B. cinerea* as *B. pseudocinerea*.

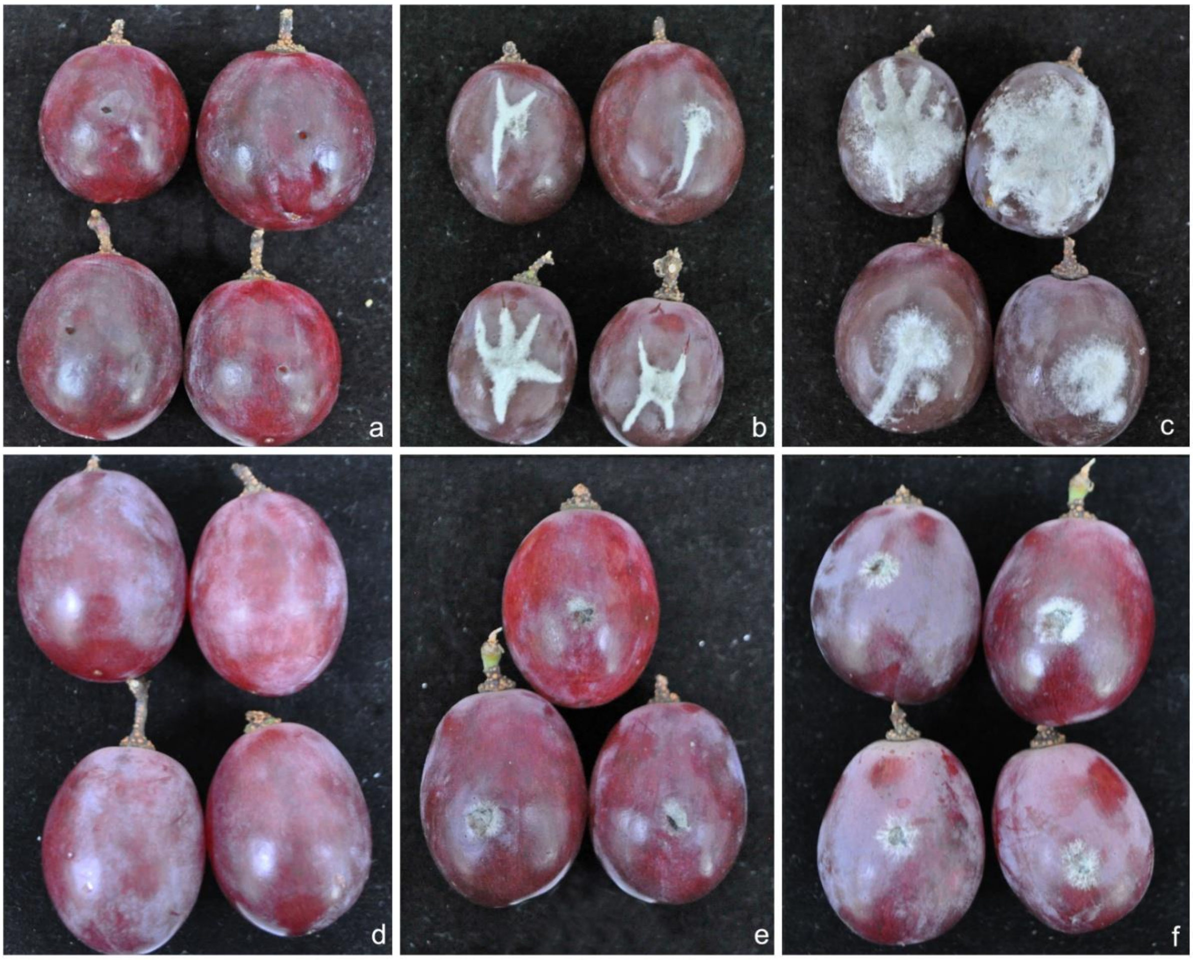

Fig. 5 – Symptoms on fruits of *V. vinifera* inoculated with *B. cinerea*. a, b, c Fruit inoculated with wounding. d, e, f Fruit inoculated without wounding. a, d Control. b, e Lesions with soft tissue and covered with ash coloured mycelium 7 days after inoculation. c, f Lesions with sunken and watery appearance 10 days after inoculation.

Table 1 Strains included in this study and their corresponding GenBank accession numbers. Ex-type strains are in bold and marked with an *, reference strains are also in bold.

Species	**Culture accession numbers** ^{1}	**Hosts**	**GenBank accession numbers**^{2}				
			RPB2	**HSP60**	**G3DPDH**	**Nep1**	**Nep2**
Botrytis aclada	MUCL8415	*Allium* spp.	AJ745664	AJ716050	AJ704992	AM087059	AM087087
Botrytis byssoidea	**MUCL94**	*Allium* spp.	AJ745670	AJ716059	AJ704998	AM087045	AM087079
Botrytis calthae	MUCL1089	*Caltha palustris*	AJ745672	AJ716061	AJ705000	AM087031$_{a}$	AM087088$_{a}$
Botrytis cinerea	**MUCL87**	>200 species	AJ745676	AJ716065	AJ705004	DQ211824$_{a}$	DQ211825$_{a}$
	JZB350001	*Vitis vinifera*	KT124647	KT021839	NA	NA	KT070547
	JZB350002	*Vitis vinifera*	NA	KT021832	KT003224	KT070527	KT070544
	JZB350003	*Vitis vinifera*	NA	KT021833	KT003226	NA	KT070552
	JZB350004	*Vitis vinifera*	KT021849	KT021828	NA	KT070517	KT070534
	JZB350006	*Vitis vinifera*	KT021860	NA	KT003232	NA	KT070548
	JZB350009	*Vitis vinifera*	KT021853	NA	KT003225	KT070521	KT070538
	JZB350010	*Vitis vinifera*	NA	KT021842	KT003238	KT070529	
	JZB350013	*Vitis vinifera*	KT021867	KT021845	KT003241	KT070526	KT070543
	JZB350016	*Vitis vinifera*	NA	KT021847	KT003243	NA	KT070549
	JZB350017	*Vitis vinifera*	KT021855	KT021834	KT003228	NA	KT070546
	JZB350018	*Vitis vinifera*	KT021865	KT021843	KT003239	KT070524	KT070541
	JZB350019	*Vitis vinifera*	KT021868	KT021846	KT003242	KT070518	KT070535
	JZB350021	*Vitis vinifera*	KT021862	NA	KT003235	KT070530	KT070556
	JZB350022	*Vitis vinifera*	KT021863	NA	KT003236	KT070519	KT070536
	JZB350023	*Vitis vinifera*	KT021866	KT021844	KT003240	NA	KT070554
	JZB350024	*Vitis vinifera*	KT021857	KT021836	KT003230	NA	KT070551
	JZB350025	*Vitis vinifera*	KT021858	KT021837	NA	KT070531	NA
	JZB350026	*Vitis vinifera*	KT021856	KT021835	KT003229	KT070525	KT070542
	JZB350027	*Vitis vinifera*	KT021848	KT021827	KT003220	NA	KT070555
	JZB350028	*Vitis vinifera*	KT021850	KT021829	KT003221	KT070522	KT070539
	JZB350029	*Vitis vinifera*	KT021851	KT021830	KT003222	KT070528	KT070545
	JZB350030	*Vitis vinifera*	KT021864	KT021841	KT003237	NA	KT070553
	JZB350035	*Vitis vinifera*	KT021852	KT021831	KT003223	KT070520	KT070537
	JZB350036	*Vitis vinifera*	KT021859	KT021838	KT003231	KT070523	KT070540
	JZB350039	*Vitis vinifera*	KT021861	NA	KT003233	KT070533	KT070557

Table 1 Continued.

Species	Culture accession numbers [1]	Hosts	GenBank accession numbers[2]				
			RPB2	**HSP60**	**G3DPDH**	**Nep1**	**Nep2**
	JZB350040	*Vitis vinifera*	KT021854	NA	KT003227	KT070532	NA
	JZB350041	*Vitis vinifera*	NA	KT021840	KT003234	NA	KT070550
Botrytis caroliniana	**CB15***=ATCC: MYA-4856	*Rubus fruticosus*	JF811590	JF811587	JF811584	JF811593	NA
Botrytis convoluta	MUCL11595	*Iris* spp.	AJ745680	AJ716069	AJ705008	AM087035	AM087062
Botrytis croci	**MUCL436**	*Crocus* spp.	AJ745681	AJ716070	AJ705009	AM087047	AM087065
Botrytis deweyae	**CBS134649***	*Hemerocallis* spp.	HG799518	HG799519	HG799521	HG799527	HG799520
Botrytis elliptica	BE9714	*Lilium* spp.	AJ745684	AJ716073	AJ705012	AM087049	AM087080
Botrytis fabae	**MUCL98**	*Vicia* spp.	AJ745686	AJ716075	AJ705014	DQ211829	DQ211831
Botrytis fabiopsis	**BC-2***=CGMCC 3.13898	*Vicia faba*	EU514473	EU514482	EU519211	NA	NA
Botrytis ficariarum	**MUCL376**	*Ficaria verna*	AJ745688	AJ716077	AJ705016	AM087055[a]	AM087085[a]
Botrytis galanthina	MUCL435	*Galanthus* spp.	AJ745689	AJ716079	AJ705018	AM087058[a]	AM087067[a]
Botrytis gladiolorum	MUCL3865	*Gladiolus* spp.	AJ745692	AJ716081	AJ705020	AM087040[a]	AM087072[a]
Botrytis globosa	MUCL444	*Allium ursinum*	AJ745693	AJ716083	AJ705022	AM087044[a]	AM087070[a]
Botrytis hyacinthi	**MUCL442**	*Hyacinthus* spp.	AJ745696	AJ716085	AJ705024	AM087048[a]	AM087066[a]
Botrytis narcissicola	MUCL2120	*Narcissus* spp.	AJ745697	AJ716087	AJ705026	AM087046	AM087078
Botrytis paeoniae	MUCL16084	*Paeonia* spp.	AJ745700	AJ716089	AJ705028	AM087032[a]	AM087064[a]
Botrytis pelargonii.	**CBS 497.50**	*Pelargonium* spp.	AJ745662	AJ716046	AJ704990	DQ211833[a]	DQ211834[a]
Botrytis polyblastis	**CBS287.38**	*Narcissus* spp.	AJ745702	AJ716091	AJ705030	AM087039	AM087074
Botrytis porri	**MUCL3234**	*Allium* spp.	AJ745704	AJ716093	AJ705032	AM087060	AM087063

Table 1 Continued.

Species	Culture accession numbers [1]	Hosts	GenBank accession numbers[2]				
			RPB2	HSP60	G3DPDH	Nep1	Nep2
Botrytis pseudocinerea	**VD110=LCP 05897**	*Vitis vinifera*	JN692428	JN692400	JN692414	NA	NA
Botrytis ranunculi	CBS178.63	*Ranunculus* spp.	AJ745706	AJ716095	AJ705034	AM087054	AM087086
Botrytis sinoallii	HMAS250008	*Allium* spp.	EU514479	EU514488	EU519217	NA	NA
Botrytis sinoviticola	**GBC-5=CGMCC 3.14977**	*Vitis vinifera*	JN692427	JN692399	JN692413	NA	NA
Botrytis sphaerosperma	MUCL21481	*Allium triquetrum*	AJ745708	AJ716096	AJ705035	AM087042	AM087068
Botrytis squamosa	MUCL1107	*Allium cepa*	AJ745710	AJ716098	AJ705037	AM087052	AM087084
Botrytis tulipae	BT9830	*Tulipa* spp.	AJ745713	AJ716102	AJ705041	AM087037	AM087077
Sclerotinia sclerotiorum	484	>400 species	AJ745716	AJ716048	AJ705044	NA	NA

[1] ATCC: American Type Culture Collection, USA; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection, China; HMAS: Institute of Microbiology, Chinese Academy of Sciences, China; JZB: Beijing Academy of Agriculture and Forestry Sciences, China; LCP: Fungal Culture Collection of the National Museum of Natural History, Paris; MUCL: Belgian Co-ordinated Collections of Micro-organisms, (agro) industrial fungi and yeasts, Belgium.

[2] G3PDH: glyceraldehydes 3-phosphate dehydrogenase; HSP60: Heat shock protein; RPB2: RNA polymerase subunit II; Nep1and Nep2: Necrosis and ethylene-inducing protein.

[a] sequences obtained from a different isolate than the one listed.

The most infective unit of *B. cinerea* for infection of grapevine in the spring are the conidia produced in late winter and early spring on over-wintering mycelium and / or sclerotia on host tissues and on the surface of soil (Elmer & Michailides 2004). It was reported that in Australia, the disease cycle is initiated by sclerotia in spring (Nair & Martin 1987, Nair & Nadtotchei 1987), but in vineyards of New Zealand sclerotia were not found (Elmer & Michailides 2004). Seyb et al. (2000a, b) identified the dominant sources of inoculum at capfall as old rachides on the ground, tendrils, leaf petioles and cane debris. Flowers of grapevine are highly susceptible to *B. cinerea* infection when they senesce (Elmer & Michailides 2004). With ripening, cuticle thickness and chemical composition of the berries change, increasing the susceptibility towards *B. cinerea* (Prudet et al. 1992, Commenil et al. 1997, Elmer & Michailides 2004). Absent or thin cuticle (Marois et al. 1986) and tight bunches increases the susceptibility of grapes to *B. cinerea* (Vail et al. 1998). *Botrytis* outbreaks in grapevine have been shown to be associated with the grape berry moth, *Lobesia botrana* (Elmer & Michailides 2004), light brown apple-moth, *Epiphyas postvittana* (Bailey et al. 1997) and *Drosophila* sp. (Capy et al. 1987).

A comprehensive phylogenetic study of the genus *Botrytis* was performed using three protein-coding genes (G3PDH, HSP60 and RPB2) supporting the morphological and host plant-based classification of *Botrytis* sp. and divided the genus into two clades (Staats et al. 2005). Clade I contained species that can only infect eudicot plants, while clade II contained species that can infect either eudicotyledonous or monocotyledonous plants. Use of these three gene regions facilitated the identification of new species in this genus (Zhang et al. 2010a, b, Li et al. 2012). Nep1 and Nep2 genes, which encode phyto-toxic proteins, have been shown to provide higher resolution in distinguishing species of *Botrytis*. These two gene regions evolved under positive selection, which suggested that these proteins have a role in the infection process (Staats et al. 2007a). These genes are proven to be neutral phylogenetic markers and adequate tools in phylogeny (Staats et al. 2007b, Cuesta et al. 2010, Hyde et al. 2014). This paper also contributes to the fact that Nep1 and Nep2 gene regions provide a higher resolution in distinguishing *Botrytis* sp.

Removal of earlier years' fruits from the trellis and fence as well as canes, bark and debris from the vineyard floor before the arrival of spring will aid in the control of Botrytis bunch rot. (Gubler et al. 1987, English et al. 1989).

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